Expression of alpha-synuclein during eye development of *Bufo arabicus*

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Alpha-synuclein contributes to neurodegenerative diseases that are characterized with the increase of its expression and collectively known as synucleinopathies such as Parkinson's disease and dementia with Lewy's bodies. A healthy balance of alpha-synuclein is important for preventing synucleinopathies, whereas the normal physiological role of alpha-synuclein is still under investigation. The present study was designed to evaluate the involvement of alpha-synuclein in the eye of *Bufo arabicus* during development, metamorphosis and adult stage. Different larval and adult stages of *Bufo arabicus* were obtained for histological and immunofluorescent expression of alpha-synuclein during eye development. The results showed that alpha-synuclein expression was associated with differentiation and migration of periocular mesenchyme cells that will form the trabecular meshwork. Also, alpha-synuclein expression was detected in the ciliary body at the adult stage. This study confirmed an additional role of alpha-synuclein during the development of the anterior eye chamber. On the other hand, synuclein expressions in *Bufo arabicus* under study helps improve our knowledge about gene conservation studies and the mechanisms by which the anterior chamber of the vertebrate eye is differentiated during development. Also, synuclein might be considered as an evolutionary conserved gene expression during eye development of *Bufo arabicus*.

Keywords: Alpha-synuclein, anterior eye chamber, eye development, trabecular meshwork.

SYNUCLEINS are small lipid-binding proteins composed of three known members α-, β- and γ-synuclein[1]. Alpha-synuclein (AS) is well known as a neurotoxic protein and its role in neurodegenerative diseases is well recognized. Synucleinopathies are characterized by proteinmisfolding, aggregation, compaction and conformational changes of AS oligomers that led to the formation of the pathological hallmark and spread of the disease in the brain. So, the dysfunction of AS becomes a factor for the development of neuropathology and synaptic dysfunction as in Parkinson's disease, dementia with Lewy bodies, multiple system atrophy and Alzheimer's disease[2–5]. Maintaining a healthy balance of AS is therefore worthwhile for preventing synucleinopathies[6]. Moreover, a lot of controversy about AS involved in the biology of normal cell functions was reported[7]. In the same scenario, the importance of AS in normal cellular function remains enigmatic[6,8]. However, in development, the level of AS increases and remains high during adulthood and participates in neuronal activity, synaptic plasticity, chaperone activity, neurofilament organization, dopamine homeostasis, vesicular transport, inhibits phospholipase D, modulates signal transduction and is age-dependent increase[6,9–11]. Also, it was recorded that AS is neuroprotective against various toxic injuries[12]. In addition, AS has been detected in erythropoietic lineage[13], the retina of vertebrates[14], lens development[15], and in the anterior chamber of the eye in some vertebrate animals[16]. Consequently, the present study focused on the involvement of AS in eye development during metamorphosis of *Bufo arabicus* to clarify the conserved gene expression of AS among vertebrate evolution, rather than its involvement in neurodegenerative pathologic diseases in higher mammalian animals.

Materials and methods

Experimental design and processing

Ten males and females of *Bufo arabicus* were collected in fertilization situation (the females carry males on their back) from valley Al-Wired, nearly 85 km from Allula governorate, Al-Madinah Al-Munawarah, Kingdom of Saudi Arabia in November 2015 where the breeding season of *B. arabicus* follows the rainfall[17]. Males and females were transferred to the laboratory and kept in an aquaria containing pond water and fed with live insects, mostly ants and flies. After a week of acclimatization, fertilized eggs were obtained by spontaneous fertilization. The adult males and females were returned to their environment. The fertilized eggs were kept separately in an aquaria containing well-aerated water that was changed three times a week. The tadpoles were fed lettuce[18]. Twenty larvae in each of the following stages were used for the larval period (20–23–25–40) and metamorphosis (42–45). The body length measurements were recorded

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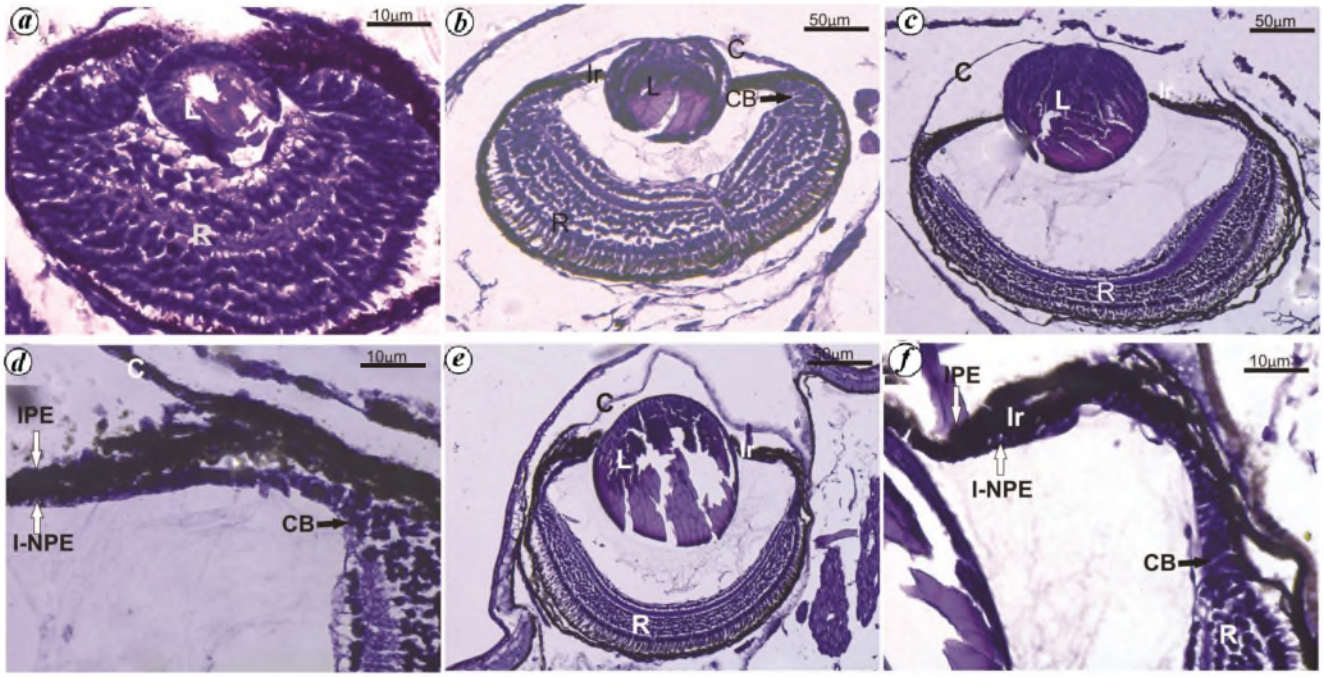

Figure 1. Photomicrograph of histological sections showing the anterior eye chamber of *Bufo arabicus* at larval stages 20 (***a***); 23 (***b***); 25 (***c***, ***d***) and 40 (***e***, ***f***) stained with haematoxylin and eosin; lens (L), retina (R), cornea (C), ciliary body (CB), iris (Ir), iris non-pigment epithelium (I-NPE) and iris pigment epithelium (IPE).

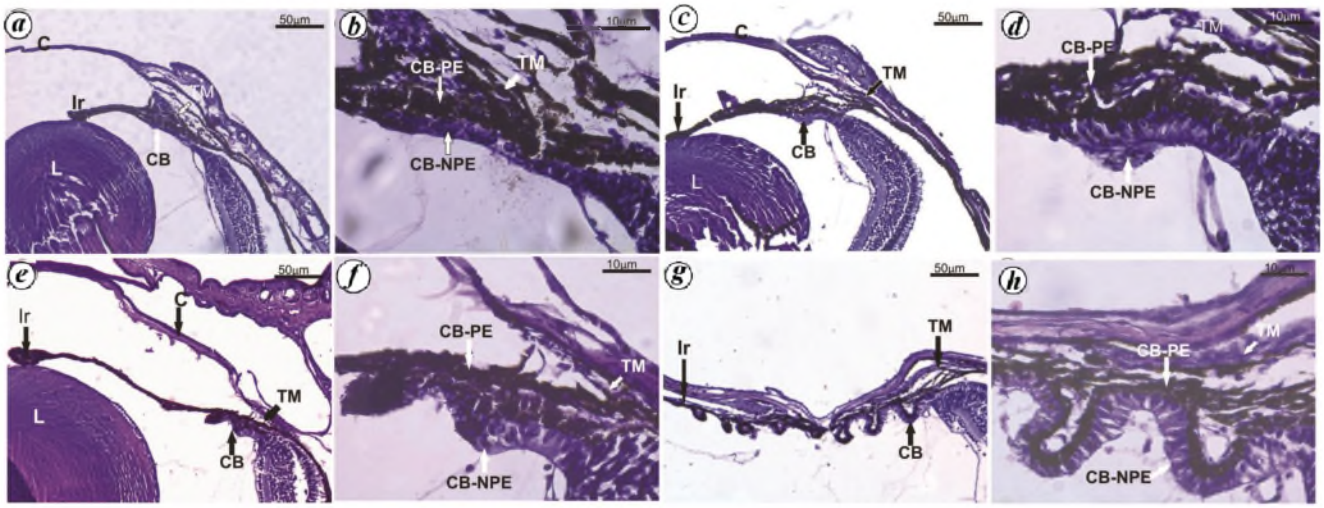

Figure 2. Photomicrographs of haematoxylin and eosin stained sections showing the anterior eye chamber of *Bufo arabicus* at metamorphosis stages 42 (***a*** and ***b***), 45 (***c*** and ***d***), adult stages young (***e*** and ***f***) and old (***g*** and ***h***); lens (L), retina (R), cornea (C), ciliary body (CB), ciliary body pigment epithelium (CB-PE), ciliary body nonpigment epithelium (CB-NPE), trabecular meshwork (TM), iris (Ir), iris non-pigment epithelium (I-NPE) and iris pigment epithelium (IPE).

for each specimen examined according to the table reported by Ba-Omar *et al.*[19]. Five small and old adults were collected from the same place. The selected studied stages were anesthetized and fixed in 10% buffered formalin, dehydrated in ascending series of ethyl alcohol, cleared in xylene and embedded in paraffin wax. The paraffin wax sections of developing eyes at studied stages at 5 µm thick were stained by haematoxylin and eosin for general histology according to Drury and Wallnigton[20].

Alpha-synuclein immunostaining technique was achieved by mounting 5 µm thick sections of developing eye at the studied stages on positive glass slides (superfrost/plus). The slides were treated with xylene for deparaffinization, descending series of ethanol for rehydration and 10 mM citrate buffer at pH 6 at 100°C for an hour to retrieve re-antigenicity[21]. The sections were blocked first with hydrogen peroxide (3%) for 10 min and then blocked with 5% bovine serum albumin (BSA) dissolved in 0.1 M phosphate buffer (pH 7.4) overnight. The sections were incubated with the primary antibody (AS, polyclonal antibody produced in rabbit, Spring Bioscience, USA) for 3 h at room temperature and then

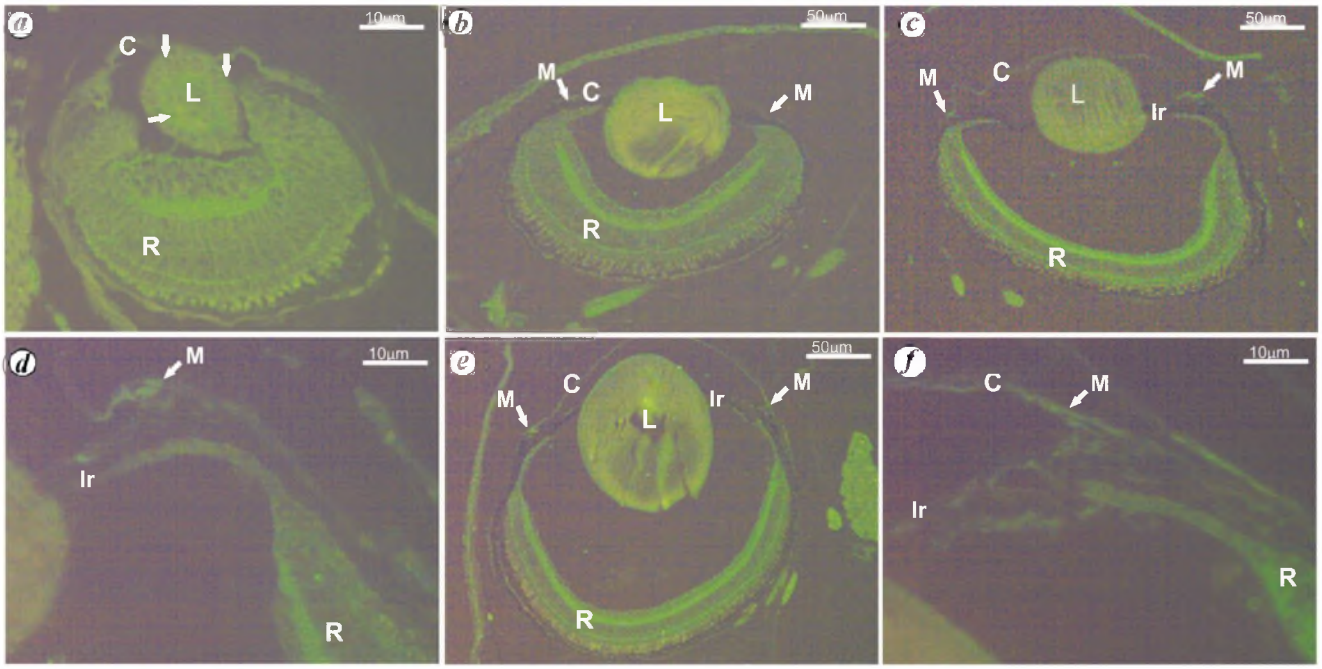

Figure 3. Photomicrographs of α-synuclein immunofluorescent stained sections showing the anterior eye chamber of *Bufo arabicus* at larval stages 20 (***a***); 23 (***b***); 25 (***c***, ***d***) and 40 (***e***, ***f***). The expression was associated with the migration and differentiation of meschenchye cells from stage 20 to 40 in trabecular meshwork region; lens (L), retina (R), meschenchyme cells (M), cornea (C), iris (Ir).

incubated with secondary antibody by dilution 1 : 1000 in phosphate buffer (FITC, goat anti-rabbit, Sigma Aldrich, USA). Washing step was achieved with phosphate buffer (pH 7.4) for three times. The sections were mounted by using vectashield (Sigma) and photographed by using the fluorescent microscope (Axio Scope A1, Zeiss, Germany). In all cases, negative control sections in which the primary antibody was not applied to tissue sections were carried out.

Results

Histological study

The general histology of the eye development in *Bufo arabicus* involved three periods: larval period (stages 20–23–25–40) (Figure 1 *a–f*), metamorphosis (stages 42–45) (Figure 2 *a–d*) and adult (young and old) stages (Figure 2 *e–h*). Stage 20 is characterized by developing lens and retina (Figure 1 *a*). Developing iris from the anterior margin of neuroepithelium of the retina was noted in stage 23 (Figure 1 *b*) and the ciliary zone refers to the region underlying the base of the iris. Increase in the length of iris (upper pigmented epithelium and lower non-pigmented epithelium) with retina differentiation was shown in stage 25 (Figure 1 *c*, *d*) and stage 40 (Figure 1 *e*, *f*). The appearance of ciliary body processes that were associated with the beginning of tail regression in the metamorphosis stages 42 (Figure 2 *a*, *b*) and 45 (Figure 2 *c*, *d*) was noted. Developing ciliary body structures that involved outer layer (pigmented epithelium) and folding in the inner layer (nonpigment epithelium) was completed either in young (Figure 2 *e*, *f*) or old adult stages (Figure 2 *g*, *h*). Pigment cells were visible along the stroma of the iris and in the anterior angle chamber (Figure 2 *a–h*). The appearance of ciliary body processes was accompanied with the development of trabecular meshwork (TM) during metamorphosis stages 42 and 45 (Figure 2 *a–d*) that developed well in the adult stages (Figure 2 *e–h*).

Immunohistochemical study

The study of AS localization during eye development of *Bufo arabicus* included three stages: larval period (20–23–25–40) (Figure 3 *a–f*), metamorphosis stages (42–45; Figure 4 *a–f*) and adult stages (young and old) (Figure 4 *g–l*). The expression of AS was noted in the outer layer of lens epithelia and in the inner bulk of lens at stage 20 (Figure 3 *a*). The anterior eye chamber was lucked with AS expression during the migration and proliferation of periocular mesenchyme cells that will form the iridocorneal angle tissue. In stage 23, the weak signal of AS was accompanied with the appearance of periocular mesenchyme cells in the iridocorneal angle (arrow, Figure 3 *b*) that gradually increase in stage 25 (arrow, Figure 3 *c*, *d*) and a bright signal of expression was pronounced in stage 40 (arrow, Figure 3 *e*, *f*). Localization of AS was associated with flattening of mesenchymal cells that will form the trabecular meshwork tissue in the metamorphosis stages 42 (arrow, Figure 4 *a*, *b*) and 45 (arrow, Figure 4 *c*, *d*). Continuous and intense expression of alpha-synuclein

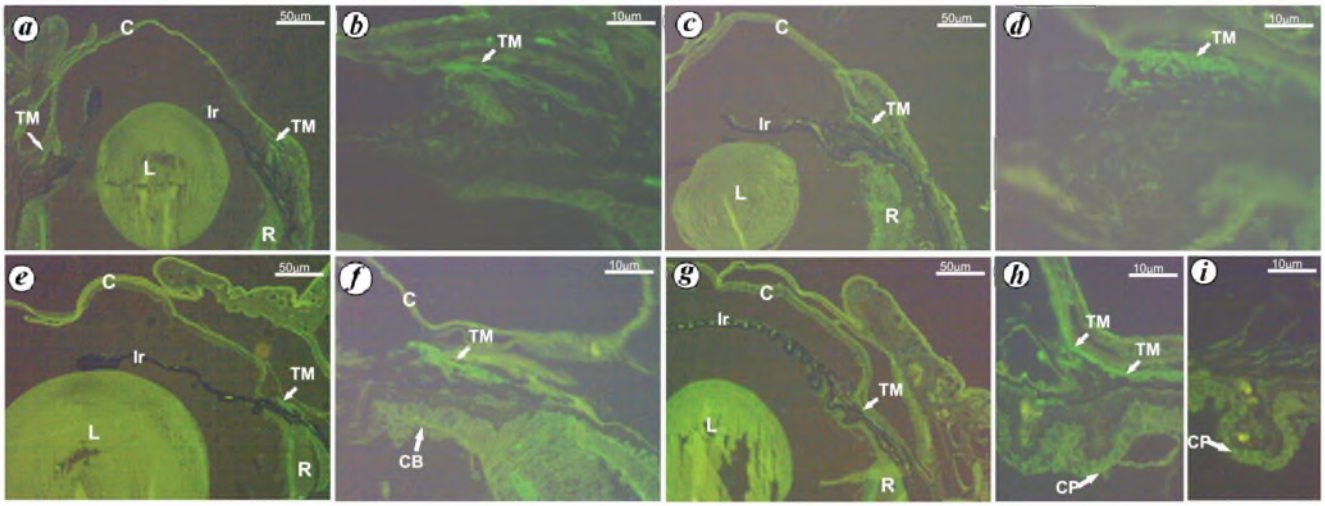

Figure 4. Photomicrographs of immunofluorescent stained sections of α-synuclein showing the anterior eye chamber development of *Bufo arabicus* at metamorphosis stages: 42 (***a*** and ***b***), 45 (***c*** and ***d***); adult stages: young (***e*** and ***f***), old (***g***, ***h*** and ***i***). The expression was pronounced in trabecular meshwork region and in ciliary body processes in adult stages; lens (L), retina (R), trabecular meshwork (TM), cornea (C), iris (Ir), ciliary body (CB), ciliary processes (CP).

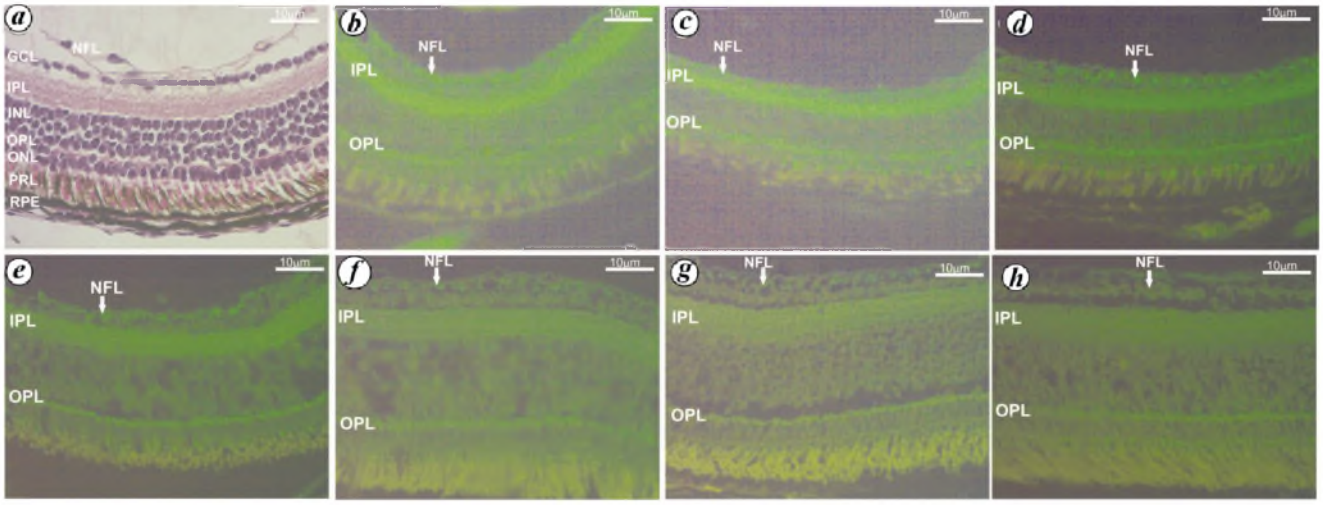

Figure 5. Photomicrographs showing the retina development of *Bufo arabicus* at larval stages 23 (***b***); 25 (***c***) and 40 (***d***); metamorphosis stages 42 (***e***), 45 (***f***); adult stages, young (***g***), old (***h***) by immunofluorescent staining of α-synuclein. The expression was pronounced in (NFL), (IPL) and (OPL). The histological structure of retina represented by haematoxylin and eosin staining at stage 25. Nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor layer (PRL) and retinal pigmented epithelial layer (RPE) (***a***).

in the trabecular meshwork region was pronounced in the young and old adult stages (arrow, Figure 4 *e*–*h*). The AS signal has been expressed in the developing inner layer of ciliary body that starts in appearance with the onset of the folding of the inner layer of the ciliary body and was clearly pronounced at adult stages (arrows, Figure 4 *f*–*i*). The retina differentiated into eight layers, nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor layer (PRL) and retinal pigmented epithelium (RPE; Figure 5 *a*). The expression of AS in the developing retina was restricted on nerve fibers (NFL), inner plexiform layer (IPL) and outer plexiform layer (OPL) through the studied stages (20, 23, 25, 40, 42, adult) (Figures 3 *a*, 5 *b*–*h*).

The gradual increase of the expression was noted from stages 20 to 25 (Figure 5 *b*, *c*). The highest expression was detected at the end of larval stage 40 (Figure 5 *d*). The gradual expression decreased from stage 40 to adult stage (Figure 5 *e*–*h*), however the lowest expression was detected in the old adult stage (Figure 5 *d*). No signals were detected in immunostaining of negative control during the differentiation of the anterior eye chamber of *Bufo arabicus* at larval stages 25 (Figure 6 *a*), 40 (Figure 6 *b*); metamorphosis stages 42 (Figure 6 *c*), 45 (Figure 6 *d*)

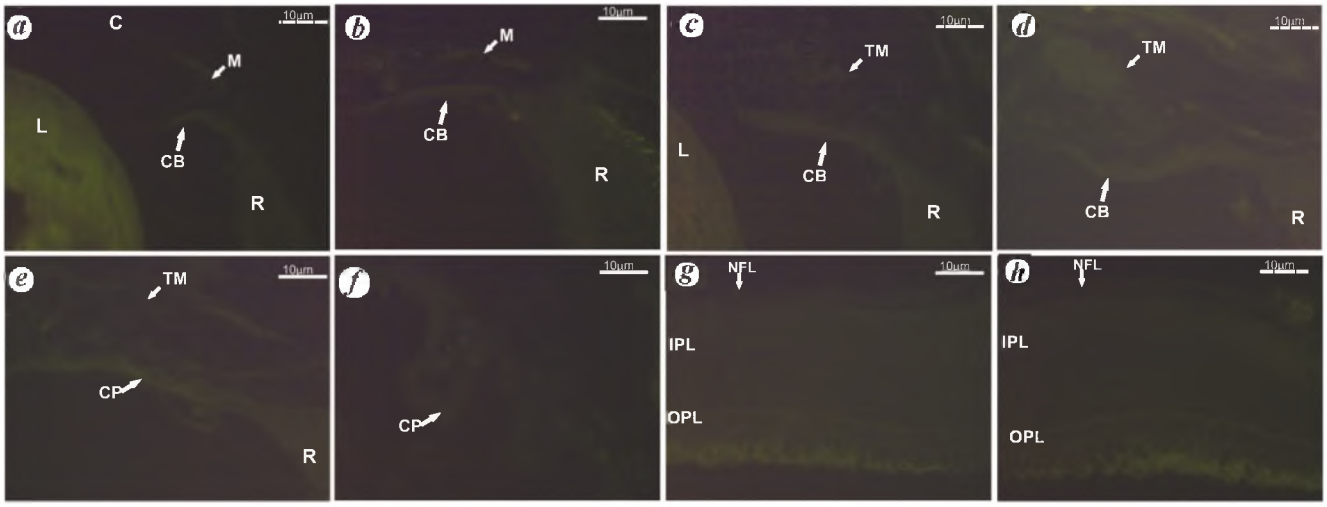

Figure 6. Photomicrographs showing the negative control staining during development of anterior eye chamber of *Bufo arabicus* at larval stages: 25 (***a***), 40 (***b***); metamorphosis stages; 42 (***c***), 45 (***d***); adult stages: young (***e***), old (***f***). Negative control staining of retina at stages 42 (***g***) and adult old (***h***).

adult stages young (Figure 6 *e*) and old (Figure 6 *f*). Also, negative staining was recorded during retinal development of *Bufo arabicus* at stages 42 (Figure 6 *g*) and adult old (Figure 6 *h*).

Discussion

The pattern of expression, localization and distribution of AS during the development of trabecular meshwork in the anterior eye chamber of *Bufo arabicus* has not been studied earlier. The present study confirms the positive localization of AS associated with the differentiation of periocular mesenchyme cells that participate in the trabecular meshwork development. In the same scenario, mesenchymal cells share in the formation of nervous system structures as well as peripheral sensory, autonomic neurons and glial cells[22,23] that were reported for positive staining of AS during migration of neurons, and synaptogenesis of the central nervous system (CNS)[24]. In addition, similar studies proved the normal physiological role of AS that has been expressed during ciliary body development of mice (*Mus musculus*), chick (*Gallus gallus domisticus*) and iridocorneal angle of the grass carp, *Ctenopharyngodon idella*[16]. Thus, AS might be inserted in the list of proteins that control the morphogenesis of the anterior eye chamber similar to PAX6 (refs 25, 26), connective tissue growth factor (CTGF), bone morphogenic proteins (BMP4 and BMP7) and transforming growth factor-β2 (TGF-β2)[27–31]. Also, it might be participating in the glaucoma disease through its role in developing the trabecular meshwork, whereas glaucoma and elevation of intraocular pressure (IOP) result from abnormal development of ocular drainage structures at the iridocorneal angle[32]. The study reveals some interesting features. Choosing of *Bufo* for the study is due to two different habitats: first, it stays its early life in aquatic habitat during the larval stages at which the ciliary body is similar to fishes[33]. Secondly, during metamorphic and adult stages accompanied with tail regression, ciliary body starts to fold completely as in adult stage of mice. Expression of AS in ciliary body processes in adult stages might be considered an accommodation for the new life in terrestrial habitat. Another difference was noted at the iridocorneal angle as in zebra fish, which stays all life in water and does not have trabecular meshwork like mammals, but has an analogous structure that is called annular ligament[33]. However, *Bufo* showed the trabecular meshwork structures like mammals that were accompanied with the ciliary body development and folding. Thirdly, the aqueous humor begins to be secreted with the developing of ciliary body that appeared in the metamorphosis and adult stages. Therefore, the mechanism of drainage of aqueous humor through iridocorneal angle during larval stages and adult stages needs more investigation.

The precise normal physiological function of AS protein is undetermined. AS distribution during developing retina examined by immunofluorescent microscopy showed the occurrence of AS in developing retinal structures as NFL, IPL and OPL in the present study. This finding is in agreement with previous studies that proved the predominant presence of AS in the IPL retina of mouse and human[7,12,34] and in outer plexiform layer[14]. In this context, AS was detected at prenatal stages E12.5, E14.5 and E16.5 in the inner nuclear layer (INL) during eye development of mice[24,35]. Moreover, AS was pronounced in the nerve fiber layer (NFL) at postnatal stages of mice[24]. In addition, Martinez-Navarrete *et al.*[14] demonstrated that the strongest expression of AS was pronounced in the IPL in African clawed toad (*Xenopus laevis*). On the other hand, abnormal accumulation of synucleins has been documented in the retinal pathology of Alzheimer's

disease, dementia with Lewy's body and glaucoma patients[34]. Also, accumulation of misfolded proteins related to retinal disease in retinitis pigmentosa and age-related macular degeneration was reported[36]. Moreover, Surguchov[1] reported that dysregulation in the synthesis of AS may damage dopaminergic retinal neurons, including amacrine and ganglion cells. The function of AS might be due to the nature of unstructured protein that occurs in a helical multimeric state on membranes and a natively unfolded state in cytosol[6,37–39]. AS has been reported in mitochondrial function[40–42]. Overexpression of AS inhibits neurotransmitter release and causes toxic effects in animal models and humans[8,43,44]. In addition, abnormalities in the dopaminergic system were detected in losing AS in knout mice[45,46]. Therefore, AS is important for the genesis, localization and/or maintenance of presynaptic vesicles[7]. In the present study, demonstration of AS was found alongside the differentiation of the anterior eye chamber and coincides with the differentiation of retinal layers. Also, AS is obviously shown to play a significant role in normal development (as a conserved gene expression); it was observed in the differentiation of iris, mesenchyme and the folds of the ciliary body. AS has a biphasic expression as revealed in both larval and adult stages of *Bufo arabicus* that may play an important role in the accommodating mechanism between aquatic and terrestrial habitats during the life cycle of the organism.

On the other side, the synuclein family is specific to the vertebrate lineage and no homologs have been found in invertebrates[47,48]. Synuclein is suggested as an evolutionarily conserved function among vertebrates[49]. A variable number of synuclein genes and encoded proteins are expressed among vertebrates depending on the species[50]. Synucleins are evolutionarily conserved in vertebrates after comparison of α-syn-coding mRNAs sequences in representative fish, amphibians, reptiles, birds and non-primate mammals[50–53]. In the lamprey, *Petromyzon marinu*, synucleins have been described as γ-syn DY, γ-syn FD and lamprey synuclein 3 on the basis of the percentage of similarity with human[54]. Pufferfish, *Takifugu rupribes* contains four types α-, β-, γ_1 and γ_2-synuclein[52]. Zebrafish, (*Danio rerio*) has β-, γ_1 and γ_2 (refs 55–57). *Xenopus laevis* has α-, β_1-, β_2- and γ-synucleins[58]. Three genes encoding for α-, β- and γ-synucleins were identified in the available genome of reptilian *Anolis carolinensis*[50] as described in birds and mammals[51,53,59]. Previous studies confirm the high degree of conservation of synuclein genes among vertebrate species. In addition, synuclein gene expression showed the prevalent expression of α- and β-syns in the central nervous system (CNS) and of γ-syns in the peripheral nervous system[51,57,58]. Moreover, similarities in the expression pattern of synuclein genes in fish *Danio rerio*[56,57], amphibians *Xenopus laevis*[58], reptilian, *Anolis carolinensis*[50] and birds *Gallus gallus*[51] suggested that synuclein may have conserved functions in nervous system development[51,57,58]. Previous studies indicate that synuclein might play an evolutionary conserved role during eye development of *Bufo arabicus*.

Conclusion

The present study provides a reference point to utilize frog species for the conserved genetic studies of AS in the mechanisms of organ differentiation as in eye development rather than the related expression in neurodegenerative diseases. The normal physiological role of AS during anterior eye chamber and retina development might be useful for further investigation.

Conflicts of interest: The authors declare no conflict of interests.

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